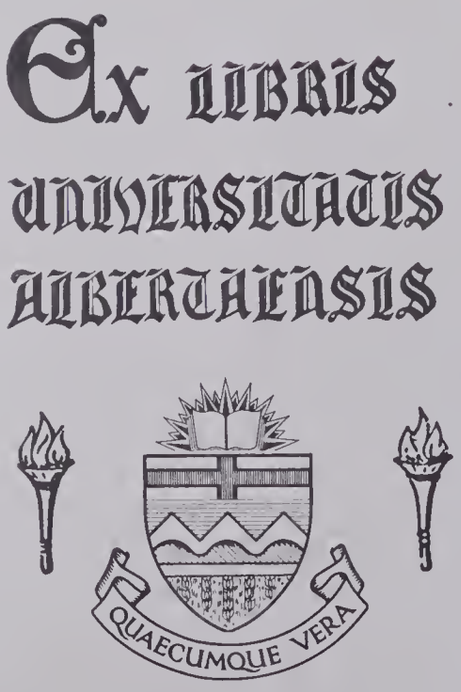

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UTILIZATION OF D-ISOMERS OF DISPENSABLE AMINO ACIDS BY THE CHICK

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled Utilization of D-isomers of Dispensable Amino Acids by the Chick submitted by Arky Yuh Chiou Du in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

Experiments were conducted to study the utilization of D-isomers of racemic dispensable amino acids by the chick. Results showed that DL-aspartic acid, DL-serine and DL-glutamic acid could be substituted isonitrogenously for L-glutamic acid in an amino acid diet at levels up to 3, 4 and 7.5%, respectively, without reducing rate of growth. At levels of 4.5, 6 and 10%, respectively, DL-aspartic acid, DL-serine and DL-glutamic acid reduced growth. Since previous studies (Renner, 1969a) using similar diets had shown that L-aspartic acid was as effective as L-glutamic acid in promoting growth, it can be concluded that the growth depression observed in the present study was due to D-aspartic acid and D-glutamic acid. Further studies are required to determine whether the growth depression observed on the addition of DL-serine was due to the D- and/or L-isomer.

Studies also showed that the addition of 4% DL-alanine but not 2% DL-alanine to the soybean protein diet reduced rate of growth. Increasing the protein content of the former helped to alleviate but did not overcome the growth inhibitory effect. Studies of the metabolic effects of supplementing the soybean protein diet with DL-alanine showed that the addition of 2 or 4% DL-alanine did not affect level of liver fat, liver nitrogen, in vitro activity of liver or kidney D-amino acid oxidase, plasma levels of L-alanine or other amino acids except D-alanine. Plasma levels of D-alanine and ammonia were significantly higher in

chicks fed diets containing 2 or 4% supplementary DL-alanine. Theoretical calculations indicated that the level of activity of D-amino acid oxidase in liver alone was sufficient to deaminate twice the amount of D-alanine consumed by chicks fed the diet containing 4% supplementary DL-alanine.

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TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Recent studies have shown that the nutritive value of some proteins can be increased by supplementation with a source of non-essential nitrogen. D-isomers of amino acids are a potential source particularly if the requirement for non-essential nitrogen is to be met by dispensable amino acids.

Information on the utilization of D-isomers of dispensable amino acids by chicks is limited. Thus, the object of the following experiments was to study the effect on chick growth of feeding diets containing graded levels of DL-alanine, DL-aspartic acid, DL-glutamic acid and DL-serine. Since previous studies in this laboratory had shown that D-alanine depresses chick growth, studies were also conducted to determine the effect of feeding graded levels of DL-alanine on plasma levels of amino acids and on the activity of liver and kidney D-amino acid oxidase. It was hoped that such studies would cast some light on why D-alanine depresses growth.

PART I

EFFECT OF D-ISOMERS OF RACEMIC DISPENSABLE AMINO ACIDS ON GROWTH OF CHICKS

Literature Review

Protein needs, whether expressed as requirements for amino acids or for nitrogen involve two components: the indispensable amino acids that must be obtained from the diet because they cannot be synthesized within the body, and non-essential nitrogen which is needed for the synthesis of dispensable amino acids and other nitrogen-containing compounds.

The importance of non-essential nitrogen for the chick was first recognized by Luckey et al. (1947). They stated that "diets containing some of the non-essential amino acids are superior to diets containing the 11 amino acids now recognized as essential for the chick".

The requirement of the chick for non-essential nitrogen was studied by Stucki and Harper (1961). They found that in order to get maximum growth on diets containing 3.64-4.24% of nitrogen about 33% of the dietary nitrogen should be supplied as dispensable amino acids if they assumed that any D-forms of indispensable amino acids present in the diet furnished indispensable nitrogen. If, on the other hand, they assumed that all D-isomers except D-methionine furnished non-essential nitrogen, then the requirement for non-essential nitrogen was 45% of the total. Recently, Sugahara and Ariyoshi (1968) calculated that in

Stucki and Harper's diet, 40% of the nitrogen came from non-essential nitrogen. For this calculation they used their data on nutritional value of D-amino acids (Sugahara et al., 1967a).

Scott and coworkers have also studied the requirement of the chick for non-essential amino acids (Greene, Scott and Johnson, 1962; Dean and Scott, 1965). In their most recent amino acid reference diet, Dean and Scott (1965) found that 12% L-glutamic acid was required for maximum growth. Calculations indicate that dispensable amino acids contributed 40% of the nitrogen. More recently, Sugahara and Ariyoshi (1968) estimated the requirement of the chick for non-essential nitrogen to be in the range of 40-50% of the nitrogen.

Studies have shown that the requirement for non-essential nitrogen is non-specific and can be met partially or completely by ammonium salts, urea, indispensable and dispensable amino acids. In this regard, Greene, Scott and Johnson (1962) observed that when the requirement for glycine and proline was met, L-glutamic acid was as effective as a mixture of glutamic acid, alanine, serine and aspartic acid in promoting growth of chicks fed diets in which nitrogen was supplied by a mixture of amino acids. Subsequently, Scott, Huston and Kelly (1966) showed that partial or complete substitution of L-glutamine for 10% L-glutamic acid in their crystalline amino acid reference diet did not improve chick growth. Other studies (Renner, 1969a) have

shown that either L-aspartic acid or L-asparagine can completely replace L-glutamic acid as a source of non-essential nitrogen without affecting chick growth. In her studies, L-alanine was not quite as effective as L-glutamic acid although equal to L-aspartic acid or L-asparagine as a source of non-essential nitrogen.

The effectiveness of urea as a source of non-essential nitrogen has been shown by Featherston, Bird and Harper (1962b) to be dependent on levels of indispensable amino acids in the diet. They observed that chicks fed amino acid diets that permitted weight gains of 4-5 g per day utilized urea to achieve growth which was as rapid, although possibly not as efficient as that achieved by the inclusion of dispensable amino acids in the diet. When the levels of indispensable amino acids in the basal diet were increased so that more rapid growth was possible urea was not as effective as the intact indispensable amino acids. This may partially explain why others have found urea to be non-effective as a source of non-essential nitrogen (Ackerson et al., 1940; Jones and Combs, 1953).

Ammonium salts also have been found to be effective as sources of non-essential nitrogen for both chicks and hens. Scott, Huston and Kelly (1966) observed that the isonitrogenous substitution of diammonium citrate for 10% L-glutamic acid reduced weight gain by 20%. In contrast, Renner (1969a) using similar diets observed that diammonium citrate was as effective as L-glutamic acid in meeting the

chicks' requirement for non-essential nitrogen. In the latter study, ammonium acetate was found to be 70-100% as effective as L-glutamic acid in promoting growth of chicks fed amino acid diets. In the case of the hen, Young et al. (1965) observed that addition of diammonium citrate to a 13% protein diet in an amount to provide the same total protein as a 16% protein diet improved egg production of hens fed the 13% protein diet up to that obtained with hens fed the 16% protein diet. Further evidence that chickens utilized both diammonium citrate and diammonium phosphate is provided by the finding of Chavez et al. (1966) that hens maintained egg production and egg size when these substances partially replaced protein in the diet.

Another potential source of non-essential nitrogen in amino acid diets is that provided by D-isomers of the indispensable amino acids which are not readily invertible. From the studies of Sugahara et al. (1967a) it would appear that for the chick the D-isomers of tryptophan, histidine, alloisoleucine, lysine, threonine and arginine which are only slightly or non-invertible are potential sources, together with 50% of the D-valine. The ability of the chick to utilize the nitrogen from D- and excess L-indispensable amino acids for the synthesis of dispensable amino acids was studied by Featherston, Bird and Harper (1962a). They fed chicks diets in which nitrogen was provided by the L-forms of indispensable amino acids in the amounts recommended by NRC (1954), at twice this level and by these same amino

acids in DL-form. Results showed that the nitrogen from D- and excess L-indispensable amino acids was utilized by the chick for synthesis of dispensable amino acids.

Information on the utilization of D-isomers of dispensable amino acids by the chick is limited. In the case of alanine, Adkins et al. (1962) observed growth depression when 2.5% DL-alanine was substituted for 2.5% L-glutamic acid in an amino acid diet for chicks. They observed that growth depression was due to the D-isomer. Sugahara et al. (1967a) observed only slight growth depression when 1% D-alanine was substituted for 1% L-alanine in an amino acid diet. In agreement with Adkins et al. (1962), Renner (1969a) found that substitution of 4.84 and 2.42% DL-alanine for ammonium acetate in an amino acid diet reduced growth by 92 and 58%, respectively. On the other hand, Anderson et al. (1951) observed only a slight growth depression when 4% DL-alanine was added to a casein-gelatin diet. In contrast, Birnbaum et al. (1957) observed that rats fed 8% D-alanine as the sole source of non-essential nitrogen still grew at 67% the rate of rats receiving a similar level of L-alanine. Sauberlich (1961) found that 5% DL-alanine added to a 6% casein-40% corn grits diet had no effect on growth of weanling rats. These results indicate that D-alanine depresses growth more in chicks than in rats.

Results of studies on the utilization of D-aspartic acid by the chick are conflicting. Sugahara et al. (1967a) showed that when 2% D-aspartic acid was substituted for 2%

L-aspartic acid in an amino acid diet for chicks, growth was depressed by 80%. The addition of 4% DL-aspartic acid to a casein-gelatin diet, however, had no effect on rate of growth (Anderson et al., 1951). In rats, Graham et al. (1950) showed that the addition of 5% DL-aspartic acid to a 20% casein diet was inhibitory while the L-form was not. Sauberlich (1961) observed that for rats 5% DL-aspartic acid was more inhibitory than 5% L-aspartic acid when added to a 6% casein diet. Increasing the level of dietary casein decreased the growth inhibitory effects, but 5% DL-aspartic acid still decreased growth even when the diet contained 34% casein.

In the case of D-serine, Sugahara et al. (1967a) have shown that replacing 1.9% L-serine by 1.9% D-serine had no effect on growth of chicks fed an amino acid diet. Renner (1969a) observed that chick growth was depressed when 4% DL-serine was substituted isonitrogenously for a portion of the ammonium acetate in an amino acid diet. Whether growth depression was due to D- and/or L-serine was not determined. Using rats as the experimental animal, Sauberlich (1961) observed only slight growth depression when 5% DL-serine was added to a 6% casein diet.

No studies have been reported on the utilization of D-glutamic acid or DL-glutamic acid by chicks. In the case of the rat, Graham et al. (1950) observed that growth was unaffected by the addition of either 5% L-glutamic acid or 5% DL-glutamic acid to a 20% casein diet.

Since information on the utilization of D-isomers of

dispensable amino acids by chicks is limited and conflicting, the following experiments were conducted to study the effect of D-isomers of racemic dispensable amino acids on growth of chicks.

Experiment 1

Adkins et al. (1962) and Renner (1969a) concluded that the decreased growth of chicks fed an amino acid diet containing 2.5 and 2.42% DL-alanine, respectively was due to the D-isomer. Since in both of these studies the amino acid mixture contained D-forms of other amino acids, the question arose as to whether the growth depression was due to D-alanine or to a general overloading of the D-amino acid oxidase system. Results of a preliminary experiment showed that the addition of 2.42% DL-alanine to a low protein diet based on soybean protein reduced growth of chicks. This study indicated that the growth depression caused by DL-alanine in the aforementioned studies was due to D-alanine and not just to a general overloading of the D-amino acid oxidase system. Since it has been shown that the effects of excesses of some indispensable amino acids can be overcome by increasing the level of dietary protein (Harper et al., 1955; Sauberlich, 1961), the following experiment was conducted to determine whether the growth inhibition caused by DL-alanine could be reduced by increasing the protein content of the diet.

Materials and Methods

The compositions of the low protein diet and the diet containing an optimum level of protein are shown in Table 1. These diets were formulated to contain 24.8 and 15.2 kcal of metabolizable energy/g of protein, respectively. Diets containing 2 and 4% supplemental DL-alanine were formulated from these diets by substituting alanine weight for weight for cornstarch.

Each diet was fed to duplicate lots of 10 female (Dominant White x White Plymouth Rock) chicks from 7-15 days of age. The chicks were housed in electrically heated, thermostatically controlled, battery brooders with raised wire-screen floors, in a temperature-controlled laboratory. The chicks were fed to 7 days of age the diet containing 15.2 kcal metabolizable energy/g protein. They were then allotted on the basis of body weight to the experimental groups and fed the experimental diets to 15 days of age. Feed and water were supplied ad libitum. Data on growth and feed consumption were obtained at intervals and feed wastage was determined daily.

Results and Discussion

Summarized in Table 2 are data showing the average daily gains, average daily feed consumptions and relative gains of chicks fed diets containing two levels of protein with and without supplemental DL-alanine.

Table 1

Composition of diets

Ingredients	Level of protein, kcal/g protein	
	15.2	24.8
	g	g
Soybean protein[1]	26.83	16.42
Glycine	0.78	0.48
DL-methionine	0.99	0.61
Cellulose[2]	5.00	5.00
Mineral mix[3]	9.25	9.25
Vitamin mix[4]	0.54	0.54
Soybean oil	15.84	15.00
Antioxidant[5]	0.025	0.025
$CaCl_2 \cdot 2H_2O$	0.207	0.207
Cornstarch	40.54	52.47

[1]Promine R - Central Soya Chemurgy Division, Chicago 39, Illinois.

[2]Solka Floc SW-40-A, Brown Forest Products Limited, Montreal, Quebec, Canada.

[3]Supplied in mg/100 g diet: $CaHPO_4 \cdot 2H_2O$, 4670; $CaCO_3$, 750; $KHCO_3$, 1900; $NaHCO_3$, 1600; $MnSO_4 \cdot H_2O$, 33; $FeSO_4 \cdot 7H_2O$, 33; $MgSO_4$, 250; KI, 0.26; $CuSO_4 \cdot 5H_2O$, 1.67; $ZnCO_3$, 11.5; $CoCl_2 \cdot 6H_2O$, 0.17; $NaMoO_4 \cdot 2H_2O$, 0.76; and Na_2SeO_3, 0.022.

[4]Supplied per 100 g diet: thiamine, 1.0 mg; riboflavin, 1.0 mg; Ca pantothenate, 4.0 mg; biotin, 0.04 mg; pyridoxine, 2.0 mg; niacin, 8.0 mg; folacin, 0.3 mg; menadione, 0.3 mg; vitamin B_{12}, 0.005 mg; choline chloride, 220 mg; inositol, 10 mg; p-aminobenzoic acid, 0.2 mg; ascorbic acid, 25 mg; vitamin A palmitate, 1000 USP units; vitamin D_3, 150 ICU; vitamin E acetate, 3.31 IU; and chlortetracycline, 1 mg.

[5]Ethoxyquin.

Table 2

Effect of level of dietary protein on weight gain and feed efficiency of chicks fed diets supplemented with DL-alanine

Treatment		Daily gain	Daily feed consumption	Growth as % of control
kcal/ g protein	Supplemental DL-alanine			
	%	g	g	
24.8	0	11.8[1,b]	20.0[a]	100[a]
	2	11.2[b]	19.0[a]	95[a]
	4	7.3[c]	14.1[b]	62[c]
15.2	0	17.0[a]	20.0[a]	100[a]
	2	16.9[a]	19.2[a]	99[a]
	4	12.4[b]	15.4[b]	73[b]

[1]Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different.

Analysis of variance and application of Duncan's multiple range test (Steel and Torrie, 1960) to the growth data showed that 2% DL-alanine added to either a low or optimum protein diet was not growth depressing. The addition of 4% DL-alanine depressed growth significantly ($P < 0.05$) irrespective of the protein content of the diet. Similar statistical treatment of the data on relative gain indicated that the addition of 4% DL-alanine was more growth depressing when chicks were fed a low protein diet than when the protein content of the diet was increased to the optimum level. Previously, Sauberlich (1961) observed that increasing the level of dietary casein decreased the growth inhibitory effects of DL-aspartic acid in rats. Harper et al. (1955) have also shown that in rats, the inhibitory effect of excess DL- or L-leucine in a low casein diet could be overcome by increasing the level of dietary protein.

Analysis of variance and application of Duncan's multiple range test (Steel and Torrie, 1960) to the data on feed consumption showed that level of dietary protein did not affect feed consumption. Feed consumption was decreased on the addition of 4% DL-alanine but chicks fed the low protein diet still consumed the same amount of feed as chicks fed the diet containing an optimum level of protein. Since chicks fed the low protein diets grew slower, they either deposited more fat or lost more energy as heat.

Experiment 2

Results of Experiment 1 indicated that supplementing a chick diet containing soybean protein as the major source of protein with 4% DL-alanine depressed chick growth. Experiment 2 was designed to determine the maximum amount of DL-alanine which could be incorporated in the diet without depressing chick growth.

Materials and Methods

Diets containing 0, 1, 2, 3, 4 and 5% supplemental DL-alanine were formulated from the low protein diet containing 24.8 kcal metabolizable energy/g protein (Table 1) by substituting DL-alanine weight for weight for cornstarch.

Each diet was fed to duplicate groups of 10 male (Dominant White x White Plymouth Rock) chicks from 7-28 days of age. The methods of allotment, feeding and housing of the chicks were as in Experiment 1. The chicks were weighed weekly and feed wastage was determined daily.

Results and Discussion

The results of this experiment (summarized in Table 3) showed that chick growth was unaffected by the addition of 1 or 2% DL-alanine to a low protein diet containing soybean protein as the major source of protein. The addition of higher levels (3, 4 or 5%) of DL-alanine caused a progressive decrease in rate of growth. A plot of weight gains versus level of dietary alanine expressed

Table 3

Weight gain and feed efficiency of chicks fed diets containing graded levels of DL-alanine (Exp. 2)

Supplemental DL-alanine	Daily gain	Feed consumed/ g gain	Growth as % of control
%	g	g	
0	14.7[1,a]	1.76[a]	100
1	14.1[a]	1.72[a]	96
2	14.2[a]	1.72[a]	97
3	12.5[b]	1.74[a]	75
4	9.9[b]	1.74[a]	67
5	8.6[c]	1.84[a]	53

[1]Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different.

as Log_{10} (0.57 + % dose) and calculation of the two lines which best fit these points using regression analysis showed that these lines intersect at 2.1% of supplemental DL-alanine. Thus, in this experiment the minimum level of supplemental DL-alanine which will reduce growth of chicks is 2.1%.

Previously, Renner (1969a) and Adkins et al. (1962) found that the incorporation of 2.42% DL-alanine and 2.5% DL-alanine, respectively, depressed the growth of chicks by 58 and 36%, respectively when incorporated in an amino acid diet. In this experiment the addition of 3% DL-alanine reduced growth by only 25%. Whether this difference in growth depression is due to the absence of other D-amino acids when nitrogen is supplied by soybean protein or to

the balance of amino acids in soybean protein is unknown.

Subsequently, a portion of this experiment was repeated (Experiment 2A) in order to obtain tissue samples and additional samples of blood plasma. The growth data obtained (Table 4) are in close agreement with the data obtained previously (Table 3).

Table 4

Weight gain and feed efficiency of chicks fed diets containing graded levels of DL-alanine (Exp. 2A)

Supplemental DL-alanine	Daily gain	Feed consumed/ g gain	Growth as % of control
%	g	g	
0	14.1[1,a]	2.03[a,b]	100
2	13.9[a]	1.92[a]	99
4	8.9[b]	2.16[b]	63

[1]Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different.

Experiment 3

Results of the preceding experiments showed that the addition of DL-alanine to semipurified diets containing soybean protein as the major source of protein reduced chick growth. The object of this experiment was to determine the effect on chick growth of the addition of DL-aspartic acid and DL-glutamic acid.

Materials and Methods

Diets containing 3% of added L-glutamic acid, DL-glutamic acid, L-aspartic acid, DL-aspartic acid or DL-alanine were formulated from the low protein diet containing 24.8 kcal metabolizable energy/g protein (Table 1) by substituting the respective amino acid weight for weight for cornstarch.

Each diet was fed to duplicate groups of 10 male (Dominant White x White Plymouth Rock) chicks from 7-26 days of age. The chicks were fed to 7 days of age on a "carbohydrate-free" diet formulated by substituting soybean oil isocalorically for cornstarch in the diet containing 15.2 kcal metabolizable energy/g protein (Table 1). The metabolizable energy value of soybean oil and cornstarch were assumed to be 9.21 (Renner, 1969a) and 4.08 (Hill, 1962) kcal/g, respectively. The methods of allotment, feeding, housing and weighing of the chicks were as in Experiment 1.

Results and Discussion

Summarized in Table 5 are data showing the average daily gains and feed efficiencies of chicks fed diets supplemented with either L-glutamic acid, DL-glutamic acid, L-aspartic acid, DL-aspartic acid or DL-alanine. Analysis of variance and application of Duncan's multiple range test (Steel and Torrie, 1960) to the data on growth showed that supplementation of the basal diet with these amino acids at

Table 5

Weight gain and feed efficiency of chicks fed the basal diet supplemented with L- or DL-amino acids

Supplement	Daily gain	Feed consumed/ g gain
	g	g
None	13.3[1,a,b]	1.94[a]
L-glutamic acid	14.4[a]	1.84[a,b]
L-aspartic acid	14.9[a]	1.86[a,b]
DL-glutamic acid	14.8[a]	1.85[a,b]
DL-aspartic acid	13.4[a,b]	1.85[a,b]
DL-alanine	12.8[b]	1.82[b]

[1]Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different.

the 3% level did not alter rate of growth. The reason why the addition of 3% DL-alanine did not reduce chick growth in this experiment but did in Experiment 2 is not apparent. The finding that the addition of 3% DL-aspartic acid did not depress growth is in agreement with the results of Anderson et al. (1951) who found that even at the 4% level, the addition of DL-aspartic acid to a casein-gelatin diet did not depress chick growth. In contrast, however, Sugahara et al. (1967a) observed that substitution of 2% D-aspartic acid for 2% L-aspartic acid in an amino acid diet depressed chick growth by 80%.

Experiment 4

This experiment was designed to determine the effect on chick growth of substituting graded levels of either DL-glutamic acid or DL-aspartic acid for L-glutamic acid in a semipurified diet in which nitrogen was supplied by a mixture of amino acids.

Materials and Methods

The composition of the basal amino acid diet used in this experiment is shown in Table 6. The amino acid mixture was patterned after that of Dean and Scott (1965) with the exception that L-glutamic acid was deleted from the mixture. When DL-forms of amino acids were used, the amino acids were included at twice the level recommended for the L-form, except for methionine. The mineral mixture was low in chloride, the chloride requirement being met by the hydrochlorides of the essential amino acids.

Diets containing graded levels of DL-glutamic acid were formulated from the basal diet by substituting DL-glutamic acid for L-glutamic acid on a weight basis. Diets containing graded levels of DL-aspartic acid were formulated by substituting DL-aspartic acid isonitrogenously for L-glutamic acid in the basal diet. Cornstarch was added in an amount to yield 100 parts of diet. The composition of the diets fed are shown in Table 7.

Table 6

Composition of basal amino acid diet

Ingredients	%
Amino acid mix[1]	14.59
Cellulose[2]	5.00
Mineral mix[3]	9.25
Vitamin mix[4]	0.56
Soybean oil	15.00
Antioxidant[5]	0.025
Chromium oxide	0.30
Cornstarch	45.27
L-glutamic acid	10.0

[1]Amino acid mixture supplied (in grams): L-lysine·HCl, 1.40; L-leucine, 1.20; DL-isoleucine, 1.60; DL-valine, 1.64; DL-methionine, 0.55; L-arginine·HCl, 1.33; L-histidine·HCl·H_2O, 0.41; DL-threonine, 1.30; L-tryptophan, 0.225; L-tyrosine, 0.63; L-cystine, 0.35; glycine, 1.60; L-proline, 1.00; and DL-phenylalanine, 1.36.

[2]Solka Floc SW-40-A, Brown Forest Products Ltd., Montreal, Quebec, Canada.

[3]See footnote 3, Table 1.

[4]See footnote 4, Table 1.

[5]Ethoxyquin.

Table 7

Composition of diets

Supplement		Constant ingredients[1]	L-glutamic acid	Cornstarch
Variable	Level			
	%	%	%	%
DL-glutamic acid	0	44.73	10.0	45.27
"	2.5	44.73	7.5	45.27
"	7.5	44.73	2.5	45.27
"	10.0	44.73	0	45.27
DL-aspartic acid	1.5	44.73	8.34	45.43
"	3.0	44.73	6.68	45.59
"	4.5	44.73	5.02	45.75
"	6.0	44.73	3.36	45.91

[1]These are all the ingredients used in the basal amino acid diet (Table 6) except for cornstarch and glutamic acid.

Each diet was fed to duplicate lots of 10 male (Dominant White x White Plymouth Rock) chicks from 7-13 days of age. The methods of allotment, feeding and housing were the same as in Experiment 1. Data on growth and feed consumption were obtained at 3 and 6 days. Feed wastage was determined daily.

Results and Discussion

Data showing the growth response and feed efficiency of chicks fed diets containing graded levels of DL-glutamic acid are summarized in Table 8.

Table 8

Weight gain and feed efficiency of chicks fed graded levels of DL-glutamic acid and DL-aspartic acid

Supplement		Daily gain	Feed consumed/ g gain
Variable	Level		
	%	g	g
DL-glutamic acid	0	12.0[1,a]	1.49[c]
"	2.5	11.7[a]	1.48[c]
"	5.0	10.9[a]	1.58[b,c]
"	7.5	9.4[a,b]	1.67[a,b,c]
"	10.0	7.6[b,c]	1.88[a]
DL-aspartic acid	1.5	9.6[a,b]	1.52[b,c]
"	3.0	9.4[a,b]	1.48[c]
"	4.5	8.0[b,c]	1.54[b,c]
"	6.0	6.1[c]	1.72[a,b]

[1]Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different.

Analysis of variance and application of Duncan's multiple range test (Steel and Torrie, 1960) to the data on growth and feed efficiency showed that both chick growth and feed efficiency were unaffected by the substitution of 2.5, 5 or 7.5% of DL-glutamic acid for a comparable amount of L-glutamic acid in the basal amino acid diet; however, the data show that the substitution of 10% DL-glutamic acid for 10% L-glutamic acid resulted in a significant depression in both growth and feed efficiency ($P<0.05$). These results indicate that the ability of the chick to utilize D-glutamic acid in amounts greater than 3.75% is limited. That rats are able to utilize low levels of DL-glutamic acid was shown by Graham et al. (1950). They found that rats fed a 20% casein diet supplemented with 5% DL-glutamic acid grew at the same rate as rats fed the casein diet supplemented with 5% L-glutamic acid.

Also summarized in Table 8 are data showing the growth and feed efficiency of chicks fed graded levels of DL-aspartic acid. The data show that the substitution of 1.5 or 3% DL-aspartic acid for an isonitrogenous amount of L-glutamic acid had no significant effect on either rate of growth or feed efficiency ($P>0.05$); however, the isonitrogenous substitution of higher levels of DL-aspartic acid (4.5 and 6%) for L-glutamic acid caused a progressive decrease in rate of growth, although only at the 6% level was feed efficiency decreased, significantly. That the growth depression observed when 4.5 and 6% DL-aspartic acid

was incorporated in the diet is due to the D-isomer is evident, since Renner (1969a) using a similar amino acid diet showed that the isonitrogenous substitution of 7.22% L-aspartic acid for 7.98% L-glutamic acid did not alter rate of growth. The extent to which DL-aspartic acid depressed growth in this experiment is markedly less than observed by Sugahara et al. (1967a). They observed that chick growth was depressed by 80% when an amino acid diet containing 2% D-aspartic acid was fed. The reason for this difference is not apparent. That DL-aspartic acid inhibits growth of rats when incorporated in diets at a level of 5% was shown by Graham et al. (1950) and Sauberlich (1961).

Results of this experiment show that chicks are able to utilize D-glutamic acid to a greater extent than D-aspartic acid when incorporated in a purified amino acid diet isonitrogenously in place of L-glutamic acid. Since Renner (1969a) using a similar diet showed that 2.42% DL-alanine depressed chick growth, it can be concluded that both D-glutamic acid and D-aspartic acid are better utilized than D-alanine.

Ratner (1944) and Wilson and Koeppe (1961) have shown that the rat, mouse and human have another D-specific enzyme in addition to D-amino acid oxidase which metabolizes D-glutamate to D-pyrrolidone carboxylate which is then excreted in the urine. Whether the chick can also convert D-glutamate to D-pyrrolidone carboxylate and whether this

explains why they can tolerate higher levels of D-glutamic acid than D-aspartic acid or D-alanine is unknown.

Experiment 5

In previous studies comparing the effectiveness of various sources of non-essential nitrogen in promoting growth of chicks fed carbohydrate-containing and "carbohydrate-free" diets, Renner (1969a) showed that DL-serine was less effective than L-glutamic acid in promoting chick growth. She also observed that chicks fed carbohydrate-containing diets containing 4% DL-serine grew better than chicks fed "carbohydrate-free" diets containing 6% DL-serine. The following experiments were conducted to determine whether the chick's response to DL-serine was affected by source of non-protein energy or whether the observed difference was due to the level fed. In addition, the effectiveness of L-serine as a source of non-essential nitrogen was determined.

Materials and Methods

Male Dominant White x White Plymouth Rock chicks were used in two duplicated experiments. In each experiment, 2 replicate groups of 10 chicks were fed each of the experimental diets from 7 to 13 days of age. The methods of allotment, feeding and housing were the same as in Experiment 1. Data on growth and feed consumption were obtained after 3 and 6 days on the experimental diets. Feed wastage was determined daily.

Diets containing 0, 2, 4 and 6% DL-serine in which non-protein energy was supplied by cornstarch were formulated from the basal amino acid diet (Table 6) by substituting DL-serine isonitrogenously for L-glutamic acid and adding cornstarch in an amount to yield 100 parts of diet. The "carbohydrate-free" counterparts were formulated by replacing starch isocalorically with soybean oil, using the values 4.08 (Hill, 1962) and 9.21 (Renner, 1969a) kcal/g for the metabolizable energy content of cornstarch and soybean oil, respectively. Extra cellulose was added to improve the texture of the diets. Because the "carbohydrate-free" diets do not total to 100, their content of non-essential nitrogen will be referred to as the level present in the carbohydrate-containing diets from which they were derived. The composition of the diets fed are shown in Table 9.

In the second experiment two replicate groups of chicks were also fed a cornstarch diet containing 6% L-serine. This diet was formulated in the same way as the diet containing 6% DL-serine. At 13 days of age these chicks were distributed on the basis of weight into 4 groups each containing 5 chicks. Two of these groups were fed a diet containing 3% L-serine while the other two groups were fed the diet containing 6% L-serine for an additional period of 6 days (13 to 19 days of age).

Table 9

Composition of diets

Source of energy	Supplemental DL-serine	Constant ingredients[1]	Cornstarch	Soybean oil	Cellulose	L-glutamic acid
	%	%	%	%	%	%
Cornstarch	0	44.725	45.37	-	-	9.91
	2	44.725	46.17	-	-	7.11
	4	44.725	46.97	-	-	4.31
	6	44.725	47.77	-	-	1.51
Soybean oil	0	44.725	-	20.74	4.66	9.91
	2	44.725	-	21.11	4.66	7.11
	4	44.725	-	21.47	4.66	4.31
	6	44.725	-	21.84	4.66	1.51

[1]These are all the ingredients used in the basal amino acid diet (Table 6) except for the cornstarch and L-glutamic acid.

Results and Discussion

Summarized in Table 10 are data showing the average daily gains and feed efficiencies of chicks fed carbohydrate-containing and "carbohydrate-free" diets containing graded levels of DL-serine. Analysis of variance and application

Table 10

Effect of supplemental DL-serine on weight gain and feed efficiency of chicks fed diets with and without carbohydrate

Treatment		Daily gain	Feed consumed/ g gain
Energy source	Supplemental DL-serine		
	%	g	g
Cornstarch	0	$10.4^{1,a}$	1.57^{c}
	2	$10.0^{a,b}$	1.55^{c}
	4	$8.7^{b,c}$	1.56^{c}
	6	3.4^{f}	2.32^{a}
Soybean oil	0	$6.9^{d,e}$	1.52^{c}
	2	$7.8^{c,d}$	1.45^{c}
	4	6.0^{e}	1.55^{c}
	6	3.8^{f}	1.84^{b}

[1]Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different.

of Duncan's multiple range test (Steel and Torrie, 1960) to the data showed that when chicks were fed either carbohydrate-containing or "carbohydrate-free" diets the isonitrogenous substitution of 2% DL-serine for L-glutamic acid had no effect on rate of growth or feed efficiency. At the 4%

level, DL-serine reduced growth of chicks fed the carbohydrate-containing diet but had no effect on growth of chicks fed the "carbohydrate-free" diet. Interpretation is difficult since in the "carbohydrate-free" series growth of chicks fed 4% DL-serine was significantly less than that of chicks fed 2% DL-serine but not less than that of chicks fed the diet containing no added DL-serine. At the 6% level, DL-serine decreased both rate of growth and feed efficiency of chicks fed diets with and without carbohydrate.

The results of a second experiment summarized in Table 11 show that the isonitrogenous substitution of 6% DL-serine for L-glutamic acid significantly decreased growth of chicks fed diets in which non-protein energy was supplied by cornstarch or soybean oil. At the 2 or 4% level, DL-serine did not affect growth or feed efficiency irrespective of source of non-protein energy. The results of this experiment indicate that source of energy does not alter the effect of DL-serine on growth. Thus, differences in level of DL-serine fed is the explanation for the difference in growth response of chicks to DL-serine when fed diets with and without carbohydrate (Renner, 1969a).

The data summarized in Table 11 also show that L-serine is less effective as a source of non-essential nitrogen than DL-serine. Previously, Sugahara and Ariyoshi (1967b) observed that when 8.06% L-serine served as the source of non-essential nitrogen in an amino acid diet, chicks lost weight. In order to determine whether the

Table 11

Effect of supplemental DL-serine and L-serine on weight gain and feed efficiency of chicks fed diets with and without carbohydrate

Treatment		Daily gain	Feed consumed/ g gain
Energy source	Supplemental DL-serine		
	%	g	g
Cornstarch	0	$10.5^{1,a}$	1.58^{b}
	2	$9.0^{a,b}$	1.67^{b}
	4	$9.0^{a,b}$	1.60^{b}
	6	3.7^{d}	2.24^{a}
Soybean oil	0	$8.5^{b,c}$	1.60^{b}
	2	$7.9^{b,c}$	1.48^{b}
	4	6.7^{c}	1.57^{b}
	6	4.4^{d}	1.65^{b}
Cornstarch	6 (L-serine)	0.1	32.95

[1]Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different.

depressed growth observed when chicks were fed 6% DL-serine was due to L-serine or D-serine, the diet of half the chicks fed 6% L-serine was changed at 13 days and for 6 days they were fed a diet containing 3% L-serine. The results obtained are summarized in Table 12. The data show that when the level of L-serine in the diet was reduced from 6% to 3% there was a marked increase in both growth and feed efficiency. Chicks fed diets containing 3% L-serine gained 11.4 g/day. In comparison, control chicks gained 10.5 g/day

Table 12

Effect of level of supplemental L-serine on weight gain and feed efficiency of chicks fed a carbohydrate-containing diet

Supplemental L-serine	Daily gain		Feed consumed/ g gain	
%	g		g	
3.0	11.5		1.30	
	11.4		1.34	
		11.4[1]		1.32
6.0	1.1		4.06	
	0.6		7.89	
		0.8		5.98

[1]Underlined values are averages of duplicate lots; individual replicate values are given in the column to the left.

during the preceding 6 days. These data show that chicks can tolerate 3% L-serine as a source of non-essential nitrogen. Thus, the growth depression observed when 6% DL-serine serves as a source of non-essential nitrogen may be due to the D-isomer. In this regard, Sugahara et al. (1967a) showed that replacing 1.9% L-serine by 1.9% D-serine had no effect on growth of chicks fed an amino acid diet. Whether replacing 3% L-serine by 3% D-serine in a chick diet will depress growth awaits further study.

PART II

METABOLIC EFFECTS OF FEEDING DL-ALANINE TO CHICKS

Literature Review

The amino acid composition of a diet can affect the pattern of amino acids in blood plasma. Studies have shown that a dietary deficiency of an amino acid results in a reduced plasma concentration of that amino acid (Kumta and Harper, 1962; Sanahuja and Harper, 1963 and Dean and Scott, 1966), whereas a dietary excess results in an accumulation of some but not all amino acids in the plasma (Hier, 1947; Sauberlich, 1961; Jones, 1964; Zimmerman and Scott, 1965; Dean and Scott, 1966; Alam et al., 1966; Sugahara and Ariyoshi, 1967b and Harper, 1968). Studies have also shown that in some cases the accumulation of an amino acid in the plasma due to a dietary excess is accompanied by changes in levels of other amino acids in the plasma (Hier, 1947; Jones, 1964; Zimmerman and Scott, 1965 and Dean and Scott, 1966).

The effect on plasma level of amino acids of feeding excessive amounts of dispensable amino acids has been studied in both rats and chicks. Sauberlich (1961) studied the effects on rats of supplementing a low protein diet containing 6% casein and 40% corn grits with 5% DL-alanine, 5% DL-aspartic acid, 5% L-aspartic acid, 5% L-glutamic acid or 5% glycine. He observed that the plasma free amino acid level increased for the specific amino acid fed in excess, but the degree of increase varied among the various amino

acids fed. For the dispensable amino acids, he found that the increase was least for L-glutamic acid and was progressively greater for glycine, L-aspartic acid, DL-alanine and DL-aspartic acid. He did not determine whether the increase was due to the D- or L-isomer. Harper (1968) also observed that glycine accumulates in the plasma of rats ingesting a diet containing excess glycine. With 4.5% of glycine in the diet, he observed that plasma glycine increased 25-fold.

In the case of chicks, Sugahara and Ariyoshi (1967b) determined plasma level of amino acids when non-essential nitrogen in an amino acid diet was provided either by a mixture of dispensable amino acids or by an isonitrogenous amount of either L-alanine, L-glutamic acid, L-aspartic acid, L-serine or glycine. The authors observed no significant accumulation of free L-glutamic acid or L-aspartic acid in the plasma when these amino acids served as the sole source of non-essential nitrogen; however, in the case of L-alanine, glycine and L-serine plasma levels increased 2.2, 8.8 and 3.7-fold, respectively. They also observed that the accumulation of glycine in the plasma of chicks was accompanied by an accumulation of serine when glycine served as the sole source of non-essential nitrogen; however, when serine served as the sole source of non-essential nitrogen plasma level of serine increased but glycine did not.

Although studies on the effect of feeding excess dispensable amino acids on plasma levels of other amino

acids are limited, many studies have been reported on the influence on plasma amino acids of feeding excesses of indispensable amino acids. Hier (1947) found that when dogs were fed excess leucine, plasma level of leucine increased, while levels of plasma arginine, methionine, phenylalanine, threonine, tyrosine and valine decreased. He also observed that when excess isoleucine was fed, plasma level of isoleucine increased while plasma levels of phenylalanine, tyrosine and valine decreased. Moreover, when excessive amounts of DL-methionine were fed, plasma level of methionine increased and plasma levels of isoleucine, phenylalanine, tyrosine and valine decreased. More recently, Jones (1964) observed using chicks that the addition of excess L-lysine to a casein-gelatin diet caused plasma and tissue levels of lysine to increase and tissue level of arginine to decrease. Dean and Scott (1966) also observed that excess dietary lysine caused plasma arginine to decrease. In addition Dean and Scott (1966) found that plasma level of glutamic acid and the mixture of plasma glutamine and asparagine decreased when excess lysine was fed. Previously, Zimmerman and Scott (1965) showed that excess of either lysine or valine in a chick diet resulted in increases in plasma threonine.

Harper (1968) has suggested that plasma amino acid concentrations are maintained at least in part by alteration in activities of amino acid degrading enzymes. In this regard, Lightbody and Kleinman (1939) and Mandelstam and Yudkin (1952) found that the activity of liver arginase in

rats increased as dietary protein increased. A similar response in activity of liver glutamic-pyruvic transaminase was reported by Rosen et al. (1959). These results have been confirmed by Muramatsu and Ashida (1962). They showed that not only did the activity of arginase and glutamic-pyruvic transaminase increase with increasing protein intake, but also the activity of glutamic-oxaloacetic transaminase increased. On the other hand, they found that the activities of D-amino acid oxidase, glutamic dehydrogenase and xanthine oxidase increased as protein intake increased up to the optimum level for growth but then showed a plateau. These results show that the effect of protein intake on enzyme activity varies and is dependent on the enzyme involved.

The effect of amino acid imbalance on enzyme activity has also been studied. Anderson et al. (1969) determined the effect of feeding a histidine-imbalanced diet on liver serine-threonine dehydratase activity of rats. Previously, the activity of this enzyme had been shown to vary directly with protein intake (Anderson et al., 1968). They found that when rats adapted to a low protein diet were fed a histidine-imbalanced diet, plasma histidine, food intake and growth decreased; total plasma amino acid concentration increased and serine-threonine dehydratase activity was low. As time progressed serine-threonine dehydratase activity increased, plasma serine plus threonine concentration fell and food intake and growth began to rise. Anderson et al. (1969)

concluded that both alterations in food intake and amino acid degrading capacity appear to contribute to the ability of rats to adjust to dietary imbalances of amino acids. The ability of rats to adjust depends on the degree of imbalance (Anderson et al., 1969) and on the protein intake (Harper, 1968).

That the addition of DL-alanine to a low protein diet based on soybean protein in amounts in excess of 2%, reduced food consumption and growth of chicks was shown in Part 1, Experiments 1 and 2. The following studies were undertaken to determine the effect of supplementary DL-alanine on plasma levels of amino acids. In addition studies were conducted to determine the effect of dietary level of DL-alanine on the activity of D-amino acid oxidase.

Materials and Methods

In the course of Experiments 2 and 2A, samples of blood, liver and kidney were taken from representative chicks at four weeks of age for determination of plasma amino acids, liver and kidney D-amino acid oxidase activity, liver fat and liver protein.

Blood samples were collected from the jugular vein of chicks using 1.5 mg sodium oxalate per ml blood to prevent coagulation. The samples were stored overnight at 5°C and centrifuged the following day at 3,000 rpm for 10 minutes.

Equal aliquots of plasma were taken from each tube, pooled according to group and stored at -24°C until analyzed.

Liver and kidney samples were taken immediately after killing chicks by dislocation of the neck. A portion of the liver was analyzed immediately for D-amino acid oxidase activity. The remaining liver was stored in plastic bags at -24°C until dried by lyophilization. The kidneys were frozen in liquid nitrogen and stored in plastic bags at -24°C until analyzed.

Preparation of blood plasma for amino acid assays

Protein-free plasma was prepared by dialyzing it against distilled water using a dialysis cell similar to that described by Hamilton and Archibald (1944).

In order to determine the proper time interval for dialysis, duplicate 3 ml portions of a solution of L-alanine (6 μg/ml) were dialyzed against 9 ml of distilled water for 3, 6, 9 and 12 hours at 5°C. Analysis of the dialyzate for L-alanine showed that the recovery of L-alanine was maximum after 9 hours. Thus, all samples were dialyzed for 9 hours.

Duplicate samples of protein-free plasma for the microbiological determination of D- and L-alanine were prepared by dialyzing 3 ml of plasma against 9 ml of distilled water for 9 hours at 5°C. Five milliliters of the dialyzate were mixed with 15 mg of freshly activated charcoal (Darco G 60 autoclaved for 1 hour at 15 pounds steam pressure) warmed and allowed to stand for 30 minutes with occasional

stirring in order to remove vitamin B_6 (Shockman, 1963). The pH was adjusted to 6.5-6.7 using KOH, diluted to 10 or 25 ml with distilled water, filtered and assayed.

Samples of protein-free plasma for assay of plasma amino acids using an automatic amino acid analyzer were prepared by dialyzing 2 ml of plasma against 5 ml of distilled water for 9 hours at 5°C. Three milliliters of the dialyzate were freeze-dried in a 5 ml vial. The residue was dissolved in 1.5-1.7 ml of a solution of HCl having a pH of 2.

Microbiological determination of L- and D-alanine

The amounts of L- and D-alanine in the samples were determined microbiologically, the media used and the assay technique followed being similar to that employed by Shockman (1963) with the exception that the levels of all amino acids in the media except glycine and L-histidine were halved (Toennies et al., 1959). Lactobacillus delbrueckii 780 (9649) was the organism used in the assay.

Assays were conducted on a semi-micro scale in which the final volume was 6 ml (Shockman, 1963). Plasma dialyzates prepared in duplicate were pipetted in duplicate into 20 x 150 mm bacteriological test tubes at levels of 0, 0.5, 1, 2 and 3 ml. Standards were pipetted in triplicate at levels of 0, 0.5, 1, 1.5, 2 and 3 ml for L-alanine (6 μg/ml), 0, 1, 2 and 3 ml for D-alanine (4 μg/ml) and 2 and 3 ml for D-alanine (8 μg/ml). Water was added to make up to 3 ml, and 3 ml basal medium were added to make a final volume

of 6 ml. The tubes were plugged with cotton, heated in the autoclave for 3 minutes at 15 pounds steam pressure, cooled, inoculated with 1 drop of inoculum and incubated at 37°C for 72 hours. The acid produced by the bacteria during growth was titrated with 0.1 N NaOH to pH 6.7 using a microburette and a pH meter.

From standard curves the amount of each amino acid present in the sample was determined.

Further details of the procedure are described in the Appendix.

Determination of plasma amino acids

One milliliter of plasma dialyzate, together with 0.1 ml of 0.2 μM norleucine as internal standard was analyzed by means of an amino acid analyzer.[1]

Determination of liver fat and liver nitrogen

Liver fat and liver nitrogen were determined on pooled samples containing 8 livers. The samples were lyophilized, the moisture content being determined by loss in weight during lyophilization.

Liver fat was determined using the method of Feigenbaum and Fisher (1963a). In this method, samples were extracted for 16 hours with a 2:1 mixture of chloroform and methanol using a Goldfisch apparatus. The dried extracts were

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[1]Technicon Auto-analyzer, Technicon Corporation, Ardsley, N.Y.

then re-extracted with light petroleum (30^{o}-60^{o}C).

Liver nitrogen was determined by macro-Kjeldahl procedure.

Determination of liver and kidney D-amino acid oxidase activity

D-amino acid oxidase activity was determined using the manometric method of Rodney and Garner (1938) as modified by Baueriedel (1962). In this method, equal amounts of liver from 2 chicks were weighed, chilled to 0^{o}C and homogenized at 0^{o}C with 9 times the tissue weight of pH 7.5 Krebs-Ringer phosphate buffer with $CaCl_2$ omitted but containing 0.05 M $NaAsO_2$. Two milliliters of the homogenate (1:10) were introduced into a Warburg flask containing 0.2 ml of 3 N NaOH and filter paper in the center well and 0.3 ml of either 0.2 M D-alanine or 0.4 M DL-alanine, DL-aspartic acid, DL-serine, DL-glutamic acid or H_2O in the side arm. The oxygen uptake was measured at 37^{o}C under an atmosphere of O_2 for a 30 minute period. The samples were run in duplicate and the uptake of O_2 attributed to the D-amino acid oxidase was calculated as μl of O_2 per hr per g of wet tissue.

Kidney samples were analyzed in the same way with the exception that the homogenate used was more dilute (1:20).

Results and Discussion

Levels of L-alanine in plasma of chicks fed diets containing graded levels of supplemental DL-alanine as determined microbiologically are summarized in Table 13.

Table 13

Concentration of L-alanine in blood plasma of chicks fed the basal diet supplemented with DL-alanine

Supplemental DL-alanine %	Plasma L-alanine, μg/ml	
	Experiment 2[1]	Experiment 2A[2]
0	66[3]	59
	83	42
	74[a]	50[b]
2	84	70
	78	61
	81[a]	66[b]
4	88	82
	78	63
	83[a]	72[b]

[1]Chicks fed experimental diets from 7-28 days of age (Exp. 2 - Table 3).

[2]Chicks fed experimental diets from 7-28 days of age (Exp. 2A - Table 4).

[3]Values represent the average of duplicate assays on pooled samples from 10 chicks. Underlined values are average values for duplicate groups. Values without a common letter in their superscript are significantly different.

Analysis of variance and application of Duncan's multiple range test (Steel and Torrie, 1960) to the data showed that supplementation of the diet with either 2 or 4% DL-alanine did not increase plasma level of L-alanine, significantly, in either of the two experiments. In contrast, Sugahara and Ariyoshi (1967b) found that when 6.83% L-alanine was substituted isonitrogenously for the dispensable amino acids in an amino acid diet for chicks,

plasma level of alanine increased 2.2 times. The failure of DL-alanine to increase plasma level of L-alanine in the present study may have been due to the low level fed.

Levels of D-alanine in plasma of chicks fed diets containing graded levels of supplemental DL-alanine as determined microbiologically are summarized in Table 14.

Table 14

Concentration of D-alanine in blood plasma of chicks fed the basal diet supplemented with DL-alanine

Supplemental DL-alanine %	Plasma D-alanine, μg/ml			
	Experiment 2[1]		Experiment 2A[2]	
0	0[3]		0	
	0		0	
		0[a]		0[a]
2	43		39	
	40		41	
		42[b]		40[b]
4	122		109	
	115		136	
		118[c]		122[c]

[1]Chicks fed experimental diets from 7-28 days of age (Exp. 2 - Table 3).

[2]Chicks fed experimental diets from 7-28 days of age (Exp. 2A - Table 4).

[3]Values represent the average of duplicate assays on pooled samples from 10 chicks. Underlined values are average values for duplicate groups. Values without a common letter in their superscript are significantly different.

Analysis of variance and application of Duncan's multiple range test (Steel and Torrie, 1960) showed that plasma levels of D-alanine increased progressively as dietary level of DL-alanine increased. Sauberlich (1961) observed a marked increase in plasma level of alanine in rats fed diets supplemented with 5% DL-alanine. He did not determine whether the increase was due to accumulation of the D- and/or L-isomer. That D-isomers may or may not accumulate in the plasma to a greater extent than L-isomers is illustrated by his findings that plasma levels of aspartic acid rose to 46 and 120 μg/ml on addition of 5% L-aspartic acid and 5% DL-aspartic acid, respectively, while, conversely, addition of 5% L-lysine and 5% DL-lysine caused plasma levels to increase to 272 and 114 μg/ml, respectively.

It is now well established that the ingestion of a diet containing a disproportionately large quantity of an amino acid can also alter the levels of other amino acids in blood plasma. In order to determine whether excess DL-alanine in the diet altered the plasma amino acid pattern of chicks, plasma amino acids were determined using an amino acid analyzer. The results obtained are summarized in Table 15.

The data show that the major effect of the addition of either 2 or 4% DL-alanine to the basal diet was to increase plasma level of alanine. A significant increase was also observed in the plasma level of ammonia. The rise in level of methionine and lysine in the plasma of chicks

Table 15

Effect of excess DL-alanine on the concentrations of free amino acids in blood plasma

Amino acid	Basal diet (A)	Basal diet + 2% DL-alanine (B)	$\frac{B-A}{A} \times 100$	Basal diet + 4% DL-alanine (C)	$\frac{C-A}{A} \times 100$
	µg/ml	µg/ml	%	µg/ml	%
Lysine	87[1]	96[1]	+10	106[1]	+ 22
Leucine	13	16	+23	15	+ 15
Isoleucine	12	12	0	12	0
Valine	14	15	+ 7	16	+ 14
Phenylalanine	17	16	- 6	18	+ 6
Tyrosine	33	30	- 9	28	- 15
Methionine	18	18	0	22	+ 22
Cystine	5	5	0	4	- 20
Arginine	58	60	+ 3	59	+ 2
Histidine	29	22	-24	29	0
Threonine & serine	111	94	-15	100	- 10
Alanine	60	98	+63	200	+233
Glycine	60	54	-10	65	+ 8
Proline	29	28	- 3	34	+ 17
Glutamic acid	29	31	+ 7	30	+ 3
Aspartic acid	14	12	-14	13	- 7
Ammonia	13	22	+69	22	+ 69

[1]Values represent the average of duplicate assays on pooled samples from 20 chicks (10/replicate group) in Exp. 2A (Table 4).

fed the basal diet supplemented with 4% DL-alanine was in excess of 20% but was not significant. In this regard it should be noted that the diet did contain supplemental DL-methionine. Previously, Marrett and Sunde (1965) observed that utilization of D-methionine for growth was dependent not only on the amount of D-amino acid present but also on the specific D-amino acid in the diet, thus indicating that under certain conditions the inversion of D-methionine might be slowed. Whether D-alanine slowed the inversion of D-methionine in the present experiment awaits further study.

For comparative purposes, plasma levels of total alanine determined using either the autoanalyzer or microbiological assay are summarized in Table 16. The data show that the total alanine content of plasma determined microbiologically is in close agreement with the values obtained using the autoanalyzer.

Results of studies on the effect of supplemental DL-alanine on activity of liver and kidney D-amino acid oxidase are summarized in Table 17.

Analysis of variance and application of Duncan's multiple range test (Steel and Torrie, 1960) to the data in Table 17 indicated that the activity of liver and kidney D-amino acid oxidase was unaffected by the level of DL-alanine fed. Muramatsu and Ashida (1962) observed that liver D-amino acid oxidase activity attained a maximal activity in rats fed a 10% casein diet and observed no

Table 16

Plasma levels of DL-alanine determined using an amino acid analyzer and microbiological assay

Supplemental DL-alanine %	Plasma level, μg/ml			
	Autoanalyzer		Microbiological	
0	63[1]		59[1,2]	
	55		42	
		59[a]		50[a]
2	111		109	
	85		102	
		98[b]		106[b]
4	202		191	
	202		199	
		202[c]		195[c]

[1]Values represent the average of duplicate assays on pooled samples from 10 chicks in Exp. 2A (Table 4). Underlined values are average values for duplicate groups. Values without a common letter in their superscript are significantly different.

[2]Values represent the sum of plasma levels of L- and D-alanine (Exp. 2A, Table 12 and Table 13).

further increase when diets containing 20, 25, 40 and 60% casein were fed. Furthermore, Bauriedel (1963) found that the feeding of 0.2% D-methionine for 3 to 4 weeks to chicks did not alter the activity of liver or kidney D-amino acid oxidase. He calculated that the observed activity of D-amino acid oxidase was sufficient to deaminate at least 2 times the amount of D-methionine which was consumed. In the present study, theoretical calculations (Table 18) indicated that the level of activity of liver D-amino

Table 17

Activity of D-amino acid oxidase in liver and kidney of chicks fed the basal diet supplemented with DL-alanine

Supplemental DL-alanine %	D-amino acid oxidase activity, μl O_2/hr/g wet tissue			
	Liver		Kidney	
0	602[1]		4125	
	443		3854	
		522		3990
2	442		3838	
	506		4706	
		474		4272
4	486		3941	
	411		3786	
		448		3864

[1]Values represent the average of duplicate determinations on 4 samples, each sample representing tissue from 2 chicks (Exp. 2A - Table 4).

acid oxidase was sufficient to deaminate twice the amount of D-alanine consumed when chicks were fed the diet containing 4% added DL-alanine. Since the activity of kidney D-amino acid oxidase was 7-9 times that of liver, the question arises as to why D-alanine accumulates in blood plasma.

Plasma levels of ammonia were observed to rise (Table 15) when either 2 or 4% DL-alanine was incorporated in the diet. Whether plasma ammonia inhibited D-amino acid oxidase activity thus permitting D-alanine to accumulate when incorporated in the diet at either the 2 or 4% level is unknown.

Table 18

Theoretical conversion of added DL-alanine to its keto analog by known D-amino acid oxidase activity

Level of DL-alanine	Feed consumed per chick per day	D-alanine consumed per chick per day	Theoretical amount of D-alanine deaminated/day		
			/g wet kidney	/g wet liver	/liver
%	g	g	g	g	g
0	30.84	0	0.79	0.12	1.44
	26.61	0	0.74	0.08	0.94
	28.7[1]	0	0.76	0.09	1.19
2	25.86	0.26	0.73	0.08	1.00
	27.24	0.27	0.90	0.10	1.45
	26.6	0.26	0.82	0.09	1.22
4	19.08	0.38	0.75	0.09	0.72
	19.12	0.38	0.72	0.08	0.74
	19.1	0.38	0.74	0.08	0.73

[1]Underlined values are average values of duplicate groups each containing 10 chicks (Exp. 2A - Table 4). Individual replicate values are given in the column to the left.

Subsequently, the rates of oxidation of equimolar amounts of DL-alanine, DL-serine, DL-glutamic acid and DL-aspartic acid by chick liver and chick kidney homogenates were determined. The data summarized in Table 19 show that chick liver homogenates oxidized DL-aspartic acid 3-4.5 times as rapidly as either DL-alanine, DL-glutamic acid or DL-serine. Chick kidney homogenates on the other hand oxidized DL-alanine most rapidly, with the oxidation of DL-serine, DL-aspartic acid and DL-glutamic acid proceeding at progressively slower rates. The finding that chick kidney

Table 19

Rate of oxidation of some dispensable DL-amino acids by D-amino acid oxidase in chick liver and kidney homogenates

Substrate	D-a.a.o. activity, μl O_2 consumed/ hr/g wet tissue	
	Liver	Kidney
DL-alanine	321[1]	3748
DL-glutamic acid	301	235
DL-aspartic acid	950	1075
DL-serine	201	1538

[1]Values represent the average of duplicate determinations on three liver or kidney homogenates.

homogenates oxidized DL-alanine and DL-aspartic acid at a greater rate than DL-glutamic acid is in agreement with results obtained by Klein and Handler (1941) using pig kidney extract. It is interesting to note that the amino acids which were most readily oxidized in vitro, i.e. DL-alanine

and DL-aspartic acid, caused the greatest growth depression while DL-glutamic acid which was slowly oxidized in vitro was least growth depressing.

Results of studies on the effect of supplemental DL-alanine on the level of liver fat and liver nitrogen are summarized in Table 20.

Table 20

Effect of supplemental DL-alanine on the level of fat and nitrogen in livers of chicks

Level of DL-alanine	Liver fat		Liver nitrogen	
%	% wet wt		% wet wt	
0	6.17[1]		2.79	
	5.91		2.52	
		6.04[a]		2.66[b]
2	6.27		2.71	
	6.44		2.89	
		6.36[a]		2.80[b]
4	6.40		2.87	
	5.36		2.64	
		5.88[a]		2.76[b]

[1]Values represent the average of duplicate determinations on pooled samples from 8 chicks (Exp. 2A - Table 4). Underlined values are average values for duplicate groups. Values without a common letter in their superscript are significantly different.

Analysis of variance and application of Duncan's multiple range test (Steel and Torrie, 1960) to the data in Table 20 showed that neither level of liver fat or liver nitrogen was affected by the level of DL-alanine fed. The effect which amino acid toxicities, imbalances and

antagonisms have on level of liver lipids in chicks has not been reported as far as the author is aware. However, chickens appear to be resistant to the development of fatty liver. Thus, in contrast to the rat increased levels of liver fat have not been observed in chicks during carbohydrate deficiency (Renner and Elcombe, 1967), starvation (Feigenbaum and Fisher, 1963a) and refeeding after starvation (Feigenbaum and Fisher, 1963b).

GENERAL DISCUSSION

The ability of the chick to utilize D-isomers of dispensable amino acids varies. Results showed that DL-aspartic acid, DL-serine and DL-glutamic acid could be substituted isonitrogenously for L-glutamic acid in an amino acid diet at levels up to 3, 4 and 7.5%, respectively, without reducing rate of growth. At levels of 4.5, 6 and 10%, respectively, DL-aspartic acid, DL-serine and DL-glutamic acid reduced growth. In comparison, 2% DL-alanine incorporated in a similar way in a similar diet depressed growth (Renner, 1969b). Since previous studies (Renner, 1969a) had shown that L-aspartic acid was as effective as L-glutamic acid in promoting growth of chicks fed similar diets, it can be concluded that the growth depression observed in the present study was due to D-aspartic acid and D-glutamic acid. Further studies are required to determine whether the growth depression observed on the addition of DL-serine was due to the D- and/or L-serine.

The reason why the ability of chicks to utilize D-forms of dispensable amino acids varies is not apparent. Differences in rate of absorption, rate of transport, rate of deamination and/or rate of excretion could influence the ability of the chick to utilize D-forms of these amino acids, thus affecting accumulation in the plasma, which in turn could affect food consumption and rate of growth. Results of in vitro studies (Table 19) showed that D-glutamic acid was the least favored substrate for deamination by D-amino acid oxidase. On the other hand, growth studies showed that it was best tolerated by the chick. Ratner (1944) and Wilson

and Koeppe (1961) have shown that the rat, mouse and human have another D-specific enzyme in addition to D-amino acid oxidase which metabolizes D-glutamate to D-pyrrolidone carboxylate which is then excreted in the urine. Whether the chick can also convert D-glutamate to D-pyrrolidone carboxylate and whether this explains why they can tolerate higher levels of D-glutamic acid than D-aspartic acid or D-alanine is unknown. The finding that chicks fed the diet containing 7.5% DL-glutamic acid plus 2.5% L-glutamic acid grew at the same rate as chicks fed 10% L-glutamic acid suggests that nitrogen from D-glutamic acid was used as a source of non-essential nitrogen. However, the question arises as to whether differences in growth response would be detected even if D-glutamic acid was not utilized and the L-glutamic acid content of the diet was thus reduced from 10 to 6.3%. Dean and Scott (1965) have reported growth of chicks fed diets containing 4 and 12% L-glutamic acid to be 12.22 and 14.89 g/chick/day, respectively, but did not indicate whether this difference was significantly different.

The extent to which D-isomers of amino acids accumulate in the serum was determined only in the case of alanine. A marked accumulation of D-alanine was observed when 4% DL-alanine was added to a diet in which nitrogen was supplied by isolated soybean protein. The accumulation of D-alanine might itself act as a signal to the satiety center resulting in a reduction in feed consumption and thus in rate of growth. On the other hand, the possibility exists that

excesses of D-alanine, or other D-amino acids, might create amino acid imbalances or antagonisms resulting in decreased food consumption and growth. This suggestion is supported by the finding that increasing the level of isolated soybean protein in the diet reduced the growth depression caused by addition of 4% DL-alanine (Part I, Exp. 1).

The degree of growth depression observed in the foregoing studies on addition of DL-alanine to a diet in which nitrogen was supplied by soybean protein was less than that observed by Renner (1969a, b) when she added DL-alanine to a diet in which nitrogen was supplied by a mixture of amino acids. The question thus arises as to whether the D-isomers of isoleucine, valine, methionine, threonine and phenylalanine present in the amino acid mixture interfered with the utilization of D-alanine or conversely did D-alanine interfere with the utilization of the D-isomers of the other amino acids. Since inversion of D-methionine was necessary to meet the chick's requirement when fed the amino acid diet but not when fed the diet based on soybean protein, the greater growth depression observed in chicks on addition of DL-alanine to the amino acid diet may have been due at least in part to their failure to convert D-methionine to L-methionine. In this regard it should be noted that LaRue et al. (1967) observed in in vitro studies using crude D-amino acid oxidase prepared from hog kidney that the deamination of one D-amino acid was inhibited by the presence of others. Moreover, Wretlind (1952) and Wachter and Berg (1960) have shown using rats that when D-methionine was

fed at a suboptimal level in an amino acid mixture it induced good growth in the absence of other D-isomers but when several D-isomers were present the growth response was impaired. Whether similar antagonisms exist in the chick is unknown, but if present might help to explain why Sugahara et al. (1967a) observed only slight growth inhibition on the substitution of D-alanine for L-alanine at the 1% level while Renner (1969b) observed a 26% reduction in chick growth when 2% DL-alanine was substituted isonitrogenously for L-glutamic acid. The amino acid diet used by Sugahara et al. (1967a) differed from the amino acid diet used in the foregoing studies in that it contained only L-amino acids. In addition levels of all amino acids in the two mixtures differed.

A significant increase in plasma ammonia was observed to accompany the rise in plasma D-alanine (Table 15). Snetsinger and Scott (1961) showed that both arginine and glycine exhibit an ability to partially overcome the growth-depressing effect of high levels of single amino acids added to an otherwise adequate diet. They postulated that glycine and arginine function in overcoming the amino acid toxicities by enhancing the excretion of excess nitrogen via the uric acid and urea cycles, respectively. Whether supplemental glycine or arginine would help to alleviate the stress induced by dietary excesses of D-amino acids in the chick is unknown.

SUMMARY

1. Results of three experiments showed that the maximum amount of DL-alanine which could be added to a soybean protein diet containing 24.8 kcal of metabolizable energy/g of protein without depressing chick growth was between 2-4%.

2. Increasing the protein content of the soybean protein diet from 24.8 kcal of metabolizable energy/g of protein to 15.2 kcal of metabolizable energy/g of protein helped to alleviate but did not overcome the growth inhibitory effect of 4% DL-alanine.

3. Results showed that DL-aspartic acid, DL-serine and DL-glutamic acid could be substituted isonitrogenously for L-glutamic acid in an amino acid diet at levels up to 3, 4 and 7.5%, respectively, without reducing rate of growth. At levels of 4.5, 6 and 10%, respectively, DL-aspartic acid, DL-serine and DL-glutamic acid reduced growth. In comparison, 2% DL-alanine incorporated in a similar way in a similar diet reduced growth by 26% (Renner, 1969b).

4. The effectiveness of DL-serine as a source of non-essential nitrogen in promoting chick growth was unaffected by whether non-protein energy was supplied by cornstarch or soybean oil.

5. Results showed that the isonitrogenous substitution of 6% L-serine for 6% DL-serine caused a marked decrease in rate of growth. A marked increase in growth rate was observed when the level of L-serine was reduced from 6 to 3%.

6. Addition of either 2 or 4% DL-alanine to the soybean protein diet containing 24.8 kcal of metabolizable energy/g of protein resulted in significant ($P < 0.05$) increases in plasma levels of D-alanine and ammonia, but did not alter plasma levels of L-alanine or other amino acids, significantly ($P > 0.05$).

7. The activity of liver and kidney D-amino acid oxidase was unaffected by the addition of 2 or 4% DL-alanine to the soybean protein diet (24.8 kcal of metabolizable energy/g of protein). Theoretical calculations indicated that the level of activity of liver D-amino acid oxidase was sufficient to deaminate twice the amount of D-alanine consumed by chicks fed the diet containing 4% added DL-alanine.

8. In vitro studies showed that chick liver homogenates oxidized DL-aspartic acid 3-4.5 times as rapidly as either DL-alanine, DL-serine or DL-glutamic acid. Chick kidney homogenates on the other hand oxidized DL-alanine most rapidly, with the oxidation of DL-serine, DL-aspartic acid and DL-glutamic acid proceeding at progressively

slower rates.

9. Results showed that neither level of liver fat or liver nitrogen was affected by the addition of 2 or 4% DL-alanine to a low protein diet.

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APPENDIX

APPENDIX

Details on the Microbiological Assay of D- and L-Alanine

Preparation of inoculum

The organism, L. delbrueckii 780 (9649), was maintained by transferring every two weeks to fresh stabs, incubating for 24 hours and then storing in the refrigerator.

To prepare inoculum the bacteria were transferred from stab to broth culture and incubated for 24 hours at 37°C. The liquid culture was then centrifuged, the supernatant decanted and the organism was washed three times with sterile water and resuspended in about 30 ml of sterile water. Aseptic technique was practised throughout.

The broth culture and stabs contained the ingredients in the amounts given in Table A-1.

Table A-1

Composition of liquid and solid broth culture media

Ingredient	Media	
	Liquid	Solid
	g	g
Proteose peptone No. 3, Difco	5.0	5.0
Bacto-yeast extract	20.0	20.0
Bacto-dextrose	10.0	10.0
Monopotassium phosphate	2.0	2.0
Sorbitan monooleate complex	0.1	0.1
Bacto-agar	---	10.0

Make up to 1,000 ml with water and tube.

Preparation of basal medium

The composition of the basal medium used in the assay of D- and L-alanine was essentially that used by Shockman (1963) with the following modifications:

1. Glutamine was added at a concentration of 1 mg/100 ml.
2. The level of biotin and folic acid was increased to double the original amount.
3. The level of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) was reduced to 3.132 g/100 ml.
4. The levels of all amino acids except glycine and L-histidine were reduced to half the amount as suggested by Toennies et al. (1959).

The composition of the stock solutions used in the preparation of the basal medium are shown in Table A-2. These solutions were stored in the refrigerator.

Table A-2

Composition of stock solutions for basal medium

1. Amino acid solution A

Glycine	32 mg
L-Histidine	3.12 mg
L-Lysine	250 mg
L-Arginine	250 mg
L-Isoleucine	125 mg
L-Leucine	125 mg
L-Methionine	125 mg
L-Proline	125 mg
L-Asparagine	125 mg
L-Threonine	125 mg
L-Valine	125 mg
L-Serine	125 mg

Make up to 50 ml with distilled water.

Table A-2 (continued)

2. Amino acid solution B

L-Aspartic acid	625	mg
L-Glutamic acid	1.25	mg
L-Phenylalanine	125	mg
D-Glutamic acid	38	mg

To dissolve add 7.5 ml of 2.5 N KOH and make up to 50 ml with distilled water.

3. L-Tyrosine

L-Tyrosine	125	mg

To dissolve add 0.75 ml of 2.5 N KOH and make up to 25 ml with distilled water.

4. Thymidine

Thymidine	16	mg

Make up to 10 ml with distilled water.

5. Mixture of K_2HPO_4 and KH_2PO_4

K_2HPO_4	5.0	g
KH_2PO_4	5.0	g

Make up to 50 ml with distilled water.

6. L-Cystine

L-Cystine	1.0	g

To dissolve add 20 ml of 2 N HCl and make up to 100 ml with distilled water.

7. L-Tryptophan

L-Tryptophan	1.0	g

Make up to 50 ml with 0.2 N HCl.

8. Riboflavin

Riboflavin	8.0	mg

Make up to 100 ml with 0.02 N acetic acid.

9. Vitamin mixture

P-Aminobenzoic acid	4	mg
Thiamin·HCl	20	mg
Ca-pantothenate	40	mg
Nicotinamide	100	mg

Make up to 250 ml with distilled water.

Table A-2 (continued)

10. Biotin

Biotin	5	mg

To dissolve add 2-3 drops NH_4OH and dilute to 50 ml with distilled water. Dilute 5 ml of this solution to 50 ml with distilled water.

11. Folic acid

Folic acid	20	mg

To dissolve add 100 ml of 0.01 N NaOH. Dilute 10 ml of this solution to 100 ml with distilled water.

12. Mixture of purines and pyrimidine

Adenine·H_2SO_4·$2H_2O$	450	mg
Guanine·HCl·H_2O	340	mg
Uracil	250	mg

To dissolve add 25 ml of 2 N HCl and make up to 250 ml with distilled water.

13. Salts

$MgSO_4 \cdot 7H_2O$	8.0	g
$MnSO_4 \cdot 4H_2O$	1.2	g
$FeSO_4 \cdot 7H_2O$	200	mg
NaCl	200	mg
$CaCl_2$	50	mg

To dissolve add 2.4 ml 1 N HCl and make up to 100 ml with distilled water.

14. Mixture of sodium oleate and Tween 40

Sodium oleate	100	mg
Tween 40	10	g

Make up to 50 ml with distilled water.

15. Glutamine

Glutamine	50	mg

Make up to 50 ml with distilled water.

Table A-2 (continued)

16. L-Alanine

L-Alanine	120	mg

To dissolve add 50 ml of distilled water and mix with 240 mg of freshly activated charcoal (Darco G 60 autoclaved for 1 hour at 15 pounds steam pressure), warm and allow to stand for 30 min with occasional stirring to remove vitamin B_6 (Shockman, 1963). Make up to 100 ml with distilled water and filter.

17. D-Alanine

D-Alanine	160	mg

Make up to 100 ml with distilled water.

The amounts of each of the stock solutions and other ingredients used in the preparation of 100 ml of basal medium are shown in Table A-3. Prior to diluting to 100 ml the medium was neutralized to pH 6.5-6.7 with KOH using a pH meter.

Table A-3

The quantity of stock solutions used to prepare 100 ml basal medium

Ingredient	Amount used	
	L-alanine	D-alanine
Amino acid solution A	4 ml	4 ml
Amino acid solution B	4 ml	4 ml
L-Tyrosine	2 ml	2 ml
Thymidine	2 ml	2 ml
Mixture of K_2HPO_4 & KH_2PO_4	1 ml	1 ml
L-Cystine	1 ml	1 ml
L-Tryptophan	0.5 ml	0.5 ml
Riboflavin	1 ml	1 ml
Vitamin mixture	1 ml	1 ml
Biotin	2 ml	2 ml
Folic acid	2 ml	2 ml
Mixture of purines & pyrimidine	2 ml	2 ml
Salts	2 ml	2 ml
Mixture of sodium oleate & Tween 40	2 ml	2 ml
Glutamine	1 ml	1 ml
L-Alanine	---	2.08 ml
D-Alanine	1.6 ml	---
Na acetate·$3H_2O$	3.132 g	3.132 g
Ascorbic acid	60 mg	60 mg
Glucose	2.0 g	2.0 g

Preparation of standards

L-alanine standard

Dilute 5 ml of a solution of L-alanine containing 1.2 mg L-alanine/ml (Table A-2, 16) to 1000 ml with distilled water. Neutralize to pH 6.5-6.7 with KOH using a pH meter.

D-alanine standards

A. Dilute 5 ml of a solution of D-alanine containing 1.6 mg D-alanine/ml (Table A-2, 17) to 1000 ml with distilled water. Neutralize to pH 6.5-6.7 with KOH using a pH meter.

B. Dilute 25 ml of solution A to 50 ml with distilled water.